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The goal of this project is to elucidate the mechanism of estrogen receptor (ER) action through the identification of proteins involved in ER signaling using a genetic approach. We have utilized the phenotype of a mutant ER (G400V ER), deficient in its ability to bind hormone, as the basis for a dosage suppression screen in yeast. Screening a high-copy yeast genomic library, we have isolated a gene that dramatically suppresses the phenotype of G400V ER by increasing its ability to bind hormone. Overexpression of this protein also increases the activity of both wild type estrogen and glucocorticoid receptors (GR). The suppressor is the yeast homologue of the human p23 protein, a component of the molecular chaperonin complex bound to many unliganded steroid receptors. The cellular distribution of p23 in yeast is mostly cytoplasmic, however, this pattern is altered upon expression of ER, whereupon p23 colocalizes with ER in the nucleus. This colocalization was seen in the absence of estrogen: addition of estrogen results in redistribution of p23 to the cytoplasm. Our work suggests that p23 is a positive regulator of the ER, and therefore a possible means by which to control ER function.

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5) Introduction

Estrogen is a steroid hormone responsible for the proper function of multiple mammalian physiological processes. In addition to its central role in reproduction, estrogen also affects the cardiovascular, skeletal, immune, and nervous systems(1,2,5). Estrogen has also been implicated in the initiation and maintenance of breast and ovarian cancers(9,10).

The estrogen signal is mediated by the estrogen receptor (ER). In the absence of estrogen, the ER is found predominantly in the nucleus, existing as a monomer bound to a number of proteins, including heat shock protein hsp 90, p23 and the immunophilins(6,7, 8). The resulting steroid-free "aporeceptor complex " inhibits ER's transcriptional activation, while keeping ER in a conformation amenable for steroid binding. Upon binding estrogen, the ER dissociates from the aporeceptor complex and dimerizes and recognizes specific DNA sequences, called estrogen response elements (ERE), within the promoter regions of estrogen responsive genes. Once bound to an ERE, the ER modulates transcription of the linked gene through interaction with the transcriptional machinery, although the exact mechanisms by which this occurs has not yet been determined(1,4).

Although many aspects of ER signaling remain to be understood, it appears that those proteins essential to ER function are conserved to such an extent among eukaryotes ER signaling can be faithfully recapitulated in yeast (*Saccharomyces cerevisiae*)(3). Yeast do not contain endogenous ER, however, the mechanism of eukaryotic gene transcription appear to be sufficiently conserved so that ER able to function in yeast in a manner analogous to that in mammalian cells. It has been established that human ER expressed in yeast can bind DNA in response to estrogen, subsequently activating transcription from EREs located in promoters upstream of reporter genes.

The genetic capabilities of yeast make it a powerful system in which to study ER function. Our overall goal is to use genetic approaches to identify proteins that affect ER function within this system to define the mechanism of signal transduction and transcriptional regulation by the receptor. We expect that the characterization of these proteins will lead to a greater understanding of the ER signal transduction pathway, and ultimately, to the identification of mammalian counterparts involved in ER function. Although the study of ER function in yeast is unlikely to lead to the understanding of the varied estrogen-induced effects seen in mammalian cells, it is a useful model for trying to determine the basic mechanism of ER signal transduction.

Factors that interact functionally with ER can be identified in yeast through dosage suppression analysis. In this approach, mutant ER proteins that display altered ability to activate transcription can be used as substrates in a dosage suppression screen to isolate

yeast genes that are capable of overcoming this discrepancy in activity. Overexpression of factors important for ER function can in principle overcome the mutational block by favoring the interactions that facilitate ER function. The advantage of this procedure is that it results in the direct cloning of genes of interest.

Using as our substrate, a mutant ER with a reduced ability to bind ligand (ER G400V), we have isolated a large genomic fragment capable of greatly increasing ER's ability to activate transcription in response to hormone. This high copy suppressor of ER is the yeast homologue of the mammalian p23 protein (yph23), a molecule previously described as a member of the aporeceptor complex of several steroid receptors. In this progress report, we provide evidence of both functional and physical interaction between the yeast p23 protein and ER. Our findings indicate that p23 is a positive regulator of the ER pathway, acting as a member of the aporeceptor complex of ER in yeast.

6) Body

Yeast Dosage Suppression Screens

One method to identify factors that affect ER function in yeast is suppressor analysis. In this approach, a mutant ER protein, displaying a reduced ability to activate transcription, is used as a substrate to isolate yeast gene product(s) that are capable of overcoming this reduced receptor activity. The simplest such strategy is dosage suppression: suppression of a mutant phenotype accomplished by overexpression of a wild type yeast gene. Overexpression of factors important for ER function can in principle overcome the receptor mutation by favoring the interaction between ER and such factors, thus reconstituting ER transcriptional activity. A major advantage of dosage suppression analysis is that it results in the direct cloning of the gene(s) that affect ER activity. Another inherent advantage of this approach is that the small size of the yeast genome requires the screening of only several thousand colonies to assay every potential yeast gene versus the millions that would be necessary to screen the larger mammalian genome. The recently completed sequencing of the yeast genome also makes possible the rapid identification of genes residing in the suppressor clone by simply matching the flanking sequences to the yeast genome database. Characterization of the isolated suppressor protein's normal function in yeast as well as in ER signaling is greatly facilitated by the relatively simple construction of yeast strains that lack the gene of interest. Thus, yeast dosage suppression analysis provides a simple and sensitive approach to the identification and isolation of factors that functionally interact with the receptor.

The ER used in the dosage suppression screen contains a mutation that replaces a glycine with a valine at residue 400 (G400V ER) within the steroid binding domain. This

receptor mutation results in decreased steroid hormone binding by ER, with a corresponding reduction in ER's ability to activate transcription at particular concentrations of estrogen. We chose this mutation as a substrate for the screen because G400V ER affects an early step in the ER pathway, namely steroid binding, and has the potential to result in the isolation of the greatest number of proteins important to ER function: factors important for either steroid binding or transcriptional activation will be capable of suppressing the G400V ER phenotype.

We tested the function of G400V ER in yeast and found that this receptor mutant exhibits a decreased ability to induce transcription when compared with wt ER, presumably due to its decreased ability to bind ligand. As seen in Figure 1, G400V ER requires a hundred-fold increase in ligand concentration before receptor transcriptional activation is observed, as compared to wt ER. At saturating ligand concentrations, however, G400V is able to reach the same maximal activity as the wt ER, suggesting that once the block to steroid binding is overcome, the receptor is able to act as efficiently as wt ER in entering the various interactions, both protein-DNA and protein-protein, that are necessary for transcriptional activation. Exploiting this phenotype, we carried out a dosage suppression screen using a high copy yeast genomic library to identify yeast proteins important for ligand binding or transcriptional activation.

The screen was carried out under conditions where yeast colonies containing the wt ER are dark blue, while the G400V ER-expressing yeast appear white (Figure 1B). Yeast strains containing G400V ER and an estrogen-responsive β-galactosidase reporter gene were transformed with a high copy yeast genomic library and assayed under these conditions. Dark blue colonies were considered to be potential suppressor candidates.

A total of 5622 transformed colonies, which represents approximately one-half of the yeast genome, were screened in an attempt to find suppressors of the G400V ER mutation. Thirteen potential suppressor colonies were isolated, but in only five was the suppression shown to be linked to the library plasmid. One library plasmid greatly exceeded the level of suppression the others exhibited, and this suppressor became the focus of our analysis. This plasmid, termed 4.3, was found to dramatically suppress the G400V ER phenotype, increasing its activity ten-fold (bringing G400V ER activity to one third the level of the wt ER) (Figure 1C). A search of the yeast genomic database, using the flanking sequences of the insert, revealed the suppressor clone to be an 8147 base pair genomic fragment with several open reading frames (ORFs) (Figure 2). At the 3' end is a truncated ORF, YKL 516, which encodes a putative protein kinase. Another, complete ORF has high homology to a known protein: the YKL522 gene is a mitochondrial ADP/ATP carrier protein homologue. However, the suppressor is most likely to be one of

the three complete open reading frames that code for unknown proteins: YKL 525, YKL520 and YKL518.

We constructed several different deletion constructs of the 4.3 plasmid in order to identify the suppressing ORF. These constructs were subsequently assayed for their ability to increase G400V ER transcription. As seen in figure 2, the suppression of the G400V ER phenotype correlated with the presence of ORF YKL 518, indicating that it was the responsible ORF.

A search of the Swissprot database revealed YKL 518 to be the yeast homologue of human p23 protein (yhp23). Although its specific function of p23 is not known, studies *in vitro* suggest that p23 is crucial to the stability of the aporeceptor complexes. Removal of p23 greatly inhibits the formation of both PR and GR aporeceptor complexes, implicating p23 as an important "organizer" of the this complex. More recent work has shown that p23 is a molecular chaperone, interacting with nonnative proteins, suppressing their aggregation, and maintaining them in an intermediate, folding-competent conformation. Thus, these studies suggest that p23 is a molecular chaperone, and may therefore function as such within the aporeceptor complex.

Affects of yeast p23 overexpression on ER and GR transcriptional activation.

Having identified the suppressing ORF as yeast p23, we constructed yeast strains that overexpress a p23 containing a HA-epitope in the presence of either G400V ER, wt ER or GR, along with a reporter plasmid containing β-galactosidase under control of the appropriate hormone response element, ERE or GRE, respectively, and measured receptor transcriptional activity as a function of yph23 overexpression. As seen in figure 4A, yeast overexpressing the HA-tagged yph23 of increases G400V ER by 4 fold, without affecting ER protein levels (Figure 3A and D). Overexpression of yph23 also increases the activity of both wt ER and GR, resulting in a greater than 50% increase in transcriptional activation for either receptor (Figure 3B and C). The ability of yph23 to functionally interact not only with the G400V ER mutant, but also with wt ER and GR, strongly implicates yph23 as a member of the normal signaling pathway of steroid receptors in yeast.

Overexpression of yeast yph23 increases ligand binding by G400V ER.

In light of p23's role in aporeceptor complex formation, and given the nature of the G400V ER mutation, we decided to examine whether suppression of the G400V ER phenotype was a result of increased ligand binding by the ER in the presence of overexpressed yph23. Steroid binding by G400V ER in the presence and absence of yph23 overexpression was measured *in vivo* (Figure 4A). Estrogen binding was assayed

by incubating both yeast strains for one hour in media containing [^{3}H]-17 β-estradiol and subsequently washed three times to remove unbound ligand. The amount of bound estradiol to G400V ER was measured by scintillation counter. As a negative control, yeast expressing GR were assayed in parallel, allowing us to determine background estradiol binding: these GR values were subtracted from the G400V ER values to determine the amount of ligand bound specifically to G400V ER.

At both 10^{-7} and 10^{-6} [^{3}H]17-β-estradiol concentrations, elevated yph23 levels resulted in an increase in estradiol binding by G400V ER (Figure 4A and 5B). At a concentration of 10^{-6} 17-β-estradiol, G400V ER bound over three times more ligand in the presence of overexpressed yph23. This pattern was more pronounced at the lower concentration of 10^{-7}, where yph23 overexpression increased ligand binding over five-fold. Western blots of the corresponding yeast cultures indicate that this result is not a function of increased receptor levels (Figure 4C). These two results would seem to suggest that the importance of yph23 to G400V ER ligand binding is inversely proportional to the concentration of hormone present. As a correlation, we might therefore expect that p23's importance to G400 V ER ligand binding would be greater at lower, more physiological, levels of hormone.

Interestingly, little effect of yph23 overexpression was seen on wt ER's ligand binding at either concentration (data not shown). We believe this is attributable to the difference in inherent binding efficiencies of the two proteins. As was shown in Figure 1, G400V ER requires a hundred-fold increase in ligand concentration to reach the same level of transcriptional activation displayed by wt ER. Correspondingly, an effect of yph23 overexpression on wt ER ligand binding might only be expected at concentrations lower than those assayed here. Consistent with this interpretation are the transcriptional assays of Figure 3B, which show that wt receptor function is enhanced by overexpression of yph23.

<u>ER and yph23 co-localize within the nucleus of yeast.</u>

Given human p23's presence in the aporeceptor complexes of PR and GR, and having shown that yph23 functionally interacts with ER within the yeast system, we proceeded to determine if yph23 and ER physically interact. We reasoned that, if yph23 is a member of the ER aporeceptor complex in yeast, then it could be expected to co-localize with ER in the nucleus. In order to determine the pattern of yph23 distribution *in vivo*, we created a yph23-GFP (green fluorescent protein) fusion protein by cloning the GFP at the extreme carboxy terminal of the yph23 protein. Importantly, this yph23-GFP fusion protein was functional and also able to suppress the G400V ER phenotype (data not

shown). The ability of the fusion protein to increase G400V ER activity establishes that the GFP moiety does not affect p23's ability to functionally interact with G400V ER.

Having established a functional interaction between yph23-GFP and G400V ER, we constructed several yeast strains which coexpressed yph23-GFP with G400V ER, wt ER, and GR. As seen in Figure 5, the distribution of yph23-GFP in the absence of any steroid receptor expression is largely cytoplasmic. Interestingly, upon coexpression of G400V ER, yph23-GFP becomes localized predominantly to the nucleus, thus co-localizing with G400V ER (Figure 5B). The same localization pattern was observed when yph23-GFP was coexpressed with wt ER (Figure 5D). Importantly, this pattern of nuclear localization was not seen when G400V ER was coexpressed with just the GFP protein, indicating that yph23 alone is responsible for the localization of the fusion protein to the nucleus (Figure 5C). Additionally, when cells coexpressing wt ER and yph23-GFP were incubated in 17-β-estradiol prior to fixation, no nuclear localization of yph23-GFP was observed: the pattern of expression in these cells resembled that of yeast expressing no ER at all, suggesting that yph23 is released upon estradiol binding (Figure 5E). As a final control, coexpression of GR, a steroid receptor that exists outside the nucleus in the unbound state, did not result in nuclear localization of yph23-GFP (Figure 5F). Instead, a more extreme cytoplasmic expression pattern was seen, where no signal corresponding to the nucleus was visible. Thus, the above-described patterns of yph23 distribution are entirely consistent with the suspected role of yph23 as a member of the aporeceptor complex of both ER and GR.

7) Conclusion

We have identified the yeast homologue of the human p23 gene as a high copy suppressor of the G400V ER phenotype. The p23 protein is a member of the steroid receptor aporeceptor complex. In yeast, overexpression of yph23 results in a greater ligand binding by G400V ER. This increase in steroid binding, in turn, results in a ten-fold increase in G400V ER-dependent transcription. This effect on activity is not limited to the mutant ER, as overexpression of yph23 was similarly shown to increase the transcriptional activity of both the wild type estrogen and glucocorticoid receptors as well. In addition to these functional assays, subcellular localization studies using a yph23-GFP fusion protein have demonstrated that yph23 relocalizes from the cytoplasm to the nucleus upon coexpression of ER, and that this nuclear localization of yph23 can be reversed by the addition of 17-β-estradiol. This nuclear re localization of yph23 was not seen on coexpression of GR, a nuclear receptor which, in the unliganded state, exists solely in the

cytoplasm. Thus, our findings demonstrate that yph23 can both functionally and physically interact with ER *in vivo* and suggets that p23 is an important component of the ER signaling pathway.

8) References

1. **Arnold, S. F., D. M. Klotz, P. M. Vonier, B. M. Collins, and J. A. McLachlan.** 1996. Synergism in estrogen-regulated gene expression. [Review] [14 refs]. *Molecular & Cellular Endocrinology* **123:**119-122.

2. **Demay, F., S. Geffroy, C. Tiffoche, M. de Monti, and M. L. Thieulant.** 1996. Cell-specific mechanisms of estrogen receptor in the pituitary gland. [Review] [45 refs]. *Cell Biology & Toxicology* **12:**317-324.

3. **Garabedian, M. J., and K. R. Yamamoto.** 1992. Genetic dissection of the signaling domain of a mammalian steroid receptor in yeast. *Molecular Biology of the Cell* **3:**1245-1257.

4. **Le Douarin, B., E. vom Baur, C. Zechel, D. Heery, M. Heine, V. Vivat, H. Gronemeyer, R. Losson, and P. Chambon.** 1996. Ligand-dependent interaction of nuclear receptors with potential transcriptional intermediary factors (mediators). [Review] [45 refs]. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences* **351:**569-578.

5. **Pelzer, T., A. Shamim, and L. Neyses.** 1996. Estrogen effects in the heart. [Review] [64 refs]. *Molecular & Cellular Biochemistry* **161:**307-313.

6. **Pratt, W. B.** 1997. The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP kinase. [Review] [183 refs]. *Annual Review of Pharmacology & Toxicology* **37:**297-326.

7. **Pratt, W. B., U. Gehring, and D. O. Toft.** 1996. Molecular chaperoning of steroid hormone receptors. [Review] [103 refs]. *Exs* **77:**79-95.

8. **Pratt, W. B., and D. O. Toft.** 1997. Steroid receptor interactions with heat shock protein and immunophilin chaperones. [Review] [698 refs]. *Endocrine Reviews* **18:**306-360.

9. **Vegeto, E., B. L. Wagner, M. O. Imhof, and D. P. McDonnell.** 1996. The molecular pharmacology of ovarian steroid receptors. [Review] [129 refs]. *Vitamins & Hormones* **52:**99-128.

10. **Wiseman, H.** 1996. Interaction of chemopreventive agents with hormone receptors. [Review] [20 refs]. *Iarc Scientific Publications* **139:**159-164.

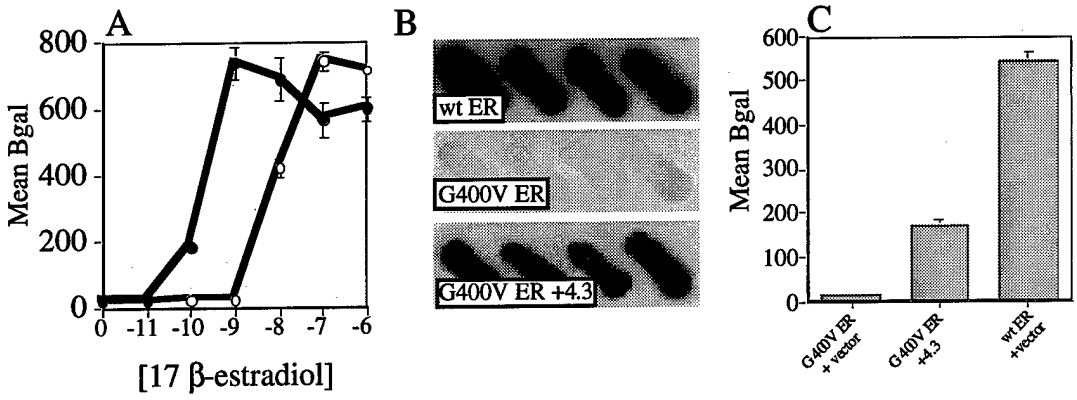

Figure 1. Dosage suppression analysis of ER (G400V).

A). Graph depicts the level of transcriptional activation exhibited by the wt ER (filled circles) and G400V ER (open circles) as a function of β-estradiol. The yeast strain W303a was transformed with 1) a galactose inducible vector expressing the wt ER and mutant G400V ER and 2) a reporter plasmid contain the β-galactosidase gene under the control of an estrogen responsive element (ERE). Cells were grown in selective media containing 2% galactose and 1% raffinose. β-estradiol was added to the media as 10^3-fold stocks in ethanol and β-galactosidase activity was determined 8 hours later. Note that G400V ER requires a hundred-fold increase in ligand concentration before receptor transcriptional activation is observed, as compared to wt ER. Exploiting this phenotype, a dosage suppression screen was performed at a β-estradiol concentration of 10^{-9}M. B). Dosage supression screen. Yeast strains containing G400V ER and an estrogen-responsive β-galactosidase reporter gene were transformed with a high copy yeast genomic library and assayed at 10^{-9} M β-estradiol, conditions where yeast colonies containing the wt ER are dark blue, while the G400V ER-expressing yeast appear white. Dark blue colonies were considered to be potential suppressor candidates. Transcriptional activity of yeast colonies containing the wt ER, ER G400V and ER G400V containing the high copy suppresser 4.3 on galactose-X-GAL indicator plates containing 10^{-9}M β-estradiol. C). Transcriptional activation of wt ER, ER G400V and ER G400V in the presence and absence of the suppresser 4.3. Wt ER and ER G400V mutant in the absence (vector) or presence of the high copy suppresser 4.3 were assayed for β-galactosidase activity in the presence 10^{-9}M β-estradiol.

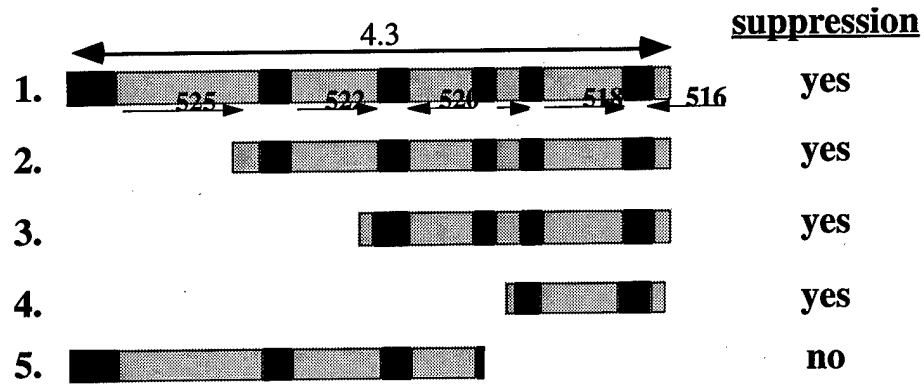

Figure 2. Suppression of ER G400V by YKL 518.
A schematic representation of a fragment of the yeast genome containing the ER G400V suppressor, 4.3. Speckled gray areas show the open reading frames (ORFs) contained within the suppressor, 4.3. Arrows denote the direction of transcription of these ORFs. Four of the genes code for complete proteins, YKL 525, YKL 522, YKL 520, YKL 518. One gene, YKL 516 is truncated at its 5' end and does not code for a protein. Deletion constructs were generated and tested for their ability to suppresses the ER G400V phenotype. ER G400V mutant in the presence of each fragment were assayed for β-galactosidase activity in the yeast strain W303a at 10^{-9}M β-estradiol. Fragments that suppressed are denoted by "Yes", whereas fragments that did not affect suppression are designated "No". Suppression of ER G400V localizes to the YKL 518 gene, which encodes the yeast homologue of the human p23 protein (yhp23).

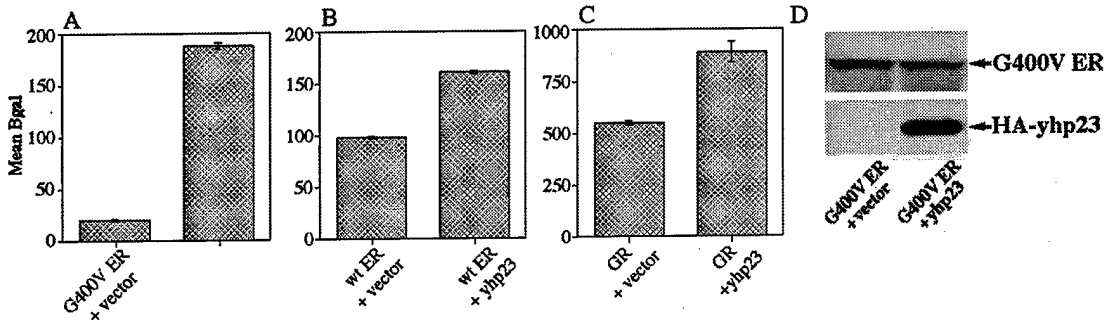

Figure 3. Effect of yhp23 overexpression on steroid receptor activity. Overexpression of yhp23 increases steroid receptor transcriptional activity. (A) ER G400V (B) wt ER, (C) and glucocorticoid receptor (GR), in the absence (vector) or presence of an HA-epitope tagged version of yhp23 in a high copy expression vector were assayed for β-galactosidase activity in the yeast strain W303a in the presence 10^{-8}M β-estradiol, for ER and 10-6M DOC for GR. D) The level of ER protein is not altered by yhp23 overexpression. Whole cell extracts were analyzed from the strain expressing ER G400V in the presence and absence of yhp23 by immunoblotting for ER, using an ER-specific rabbit polyclonal antiserum (top panel), and for HA-tagged yhp23, using anti-HA mouse monoclonal antibodies (bottom panel).

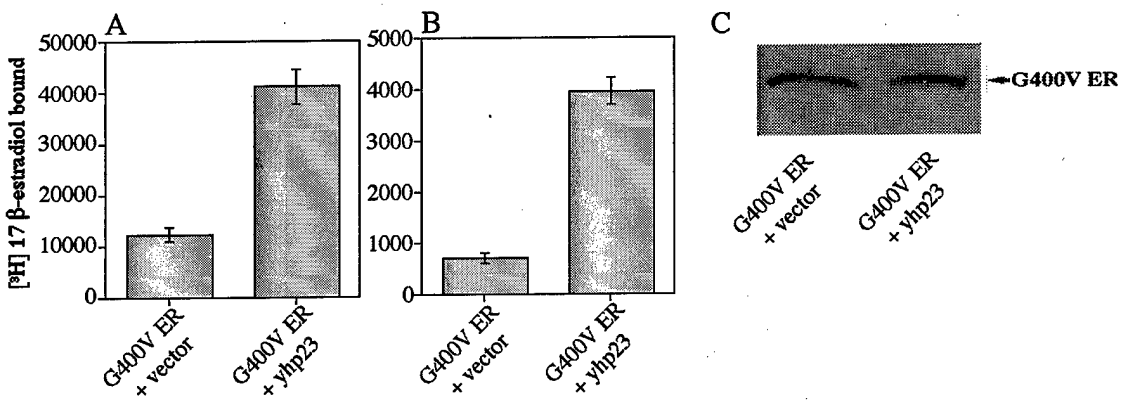

Figure 4. Overexpression of yhp23 increases steroid binding by G400V ER *in vivo*. A) Steroid binding *in vivo* by G400V ER in the presence and absence of yhp23. Estrogen binding was assayed by incubating yeast strains containing G400V ER for one hour in media containing (A) 10^{-7} and (B) 10^{-6} [^{3}H]17-β-estradiol. Cells were subsequently washed three times to remove unbound ligand and the amount of bound [^{3}H]17-β-estradiol was measured by scintillation counter. To determine background estradiol binding, yeast expressing GR were assayed in parallel: these GR values were subtracted from the G400V ER values to determine the amount of [^{3}H]17-β-estradiol bound specifically to G400V ER. C). Protein levels of G400V ER are not affect by yhp23 overexpression. Whole cell extracts were analyzed from strain expressing ER G400V in the presence (vector) and absence of yhp23 (yhp23) by immunoblotting for ER, using an ER-specific rabbit polyclonal antiserum.

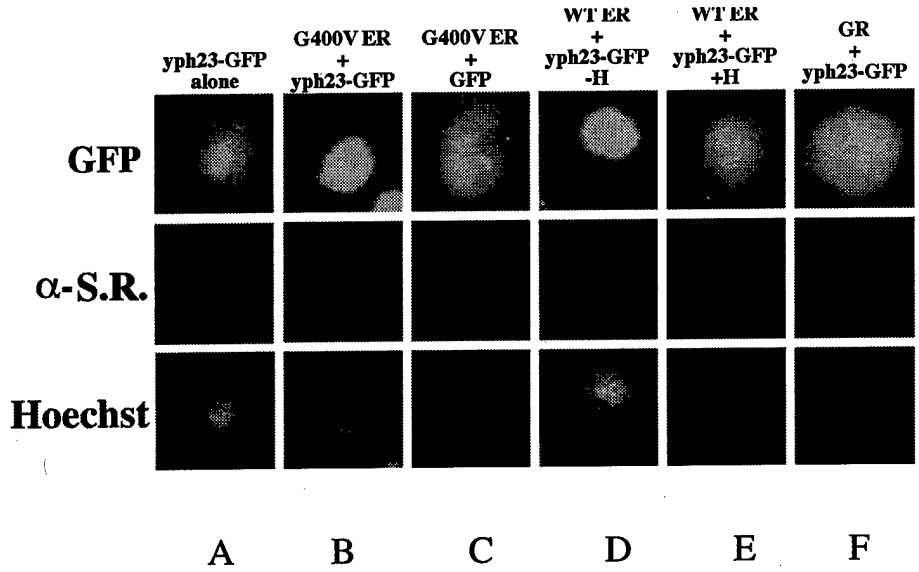

Figure 5. Co-localization of ER and yhp23.
To visualize yhp23 *in vivo*, a yhp23-green fluorescent protein (GFP) fusion protein was created by placing the GFP moiety onto the carboxy terminus of yhp23 (yhp23-GFP). The yeast strain W303a was transformed with a yhp23-GFP expression vector or GFP alone along with a galactose inducible vector expressing the wt ER and mutant G400V ER or GR. Cells were treated with 10^{-8}M β-estradiol (H+) or mock treated with ethanol vehicle (-H) and the distribution of GFP, ER or GR (α-SR) and the nucleus (Hoechst) were determined by fluorescent microscopy using a Zeiss Axioplan Fluorescent Microscope. A) The distribution of p23-GFP in the absence of any steroid receptor expression is largely cytoplasmic. B) Upon coexpression of G400V ER, p23-GFP becomes localized predominantly to the nucleus. C) Nuclear localization of GFP only was not seen when G400V ER was coexpressed, indicating that p23 is responsible for the localization of the fusion protein to the nucleus. D) Nuclear localization of yhp23-GFP was observed when yhp23-GFP was coexpressed with wt ER. E) No nuclear localization of yhp23-GFP was observed, when cells coexpressing wt ER and p23-GFP were incubated in 17-β-estradiol: the pattern of yhp23-GFP expression in these cells resembled that of yeast expressing no ER at all, suggesting that yhp23 is released upon estradiol binding. F) Coexpression of GR, a steroid receptor that exists outside the nucleus in the unbound state, did not result in nuclear localization of yhp23-GFP.